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SCREENING OF FACTORS INFLUENCING EXO-POLYGALACTURONASE PRODUCTION BY *ASPERGILLUS NIGER* ATCC 120120 USING TWO-LEVEL FRACTIONAL FACTORIAL DESIGN

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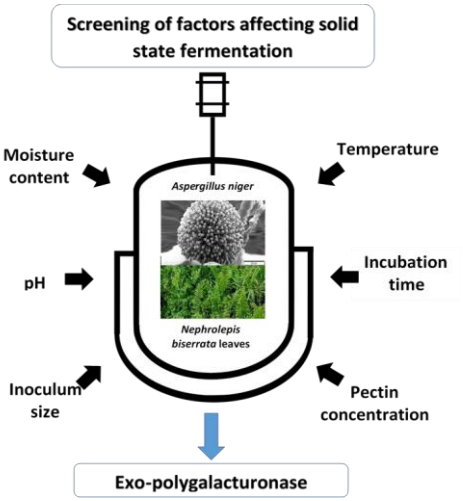
Graphical abstract



Abstract

Exo-polygalacturonase was produced by *Aspergillus niger* ATCC 120120 in a solid-state fermentation using *Nephrolepis biserrata* leaves. Factors affecting the production of exo-polygalacturonase were determined using a two-level fractional factorial design. The screening process for six factors; pH, incubation time, temperature, pectin concentration, inoculum size and moisture content, that influence the production of exo-polygalacturonase by *A. niger* was performed. The result of variance analysis (ANOVA) suggested that there were four statistically significant ($P < 0.005$) factors in the production of exo-polygalacturonase by *A. niger*. These factors were incubation time, temperature, pectin concentration and moisture content. The statistical analysis shows that the linear mathematical model is significant with coefficient of determination (R^2) value of 0.9711. The optimum production of exo-polygalacturonase obtained using the model in this study was at 40.00 U/g.

Keywords: *Aspergillus niger*, exo-polygalacturonase, two-level factorial design, pectinase

Abstrak

Ekso-poligalakturonase telah dihasilkan oleh *Aspergillus niger* ATCC 120120 melalui fermentasi keadaan pepejal menggunakan daun *Nephrolepis biserrata*. Faktor-faktor yang mempengaruhi penghasilan ekso-poligalakturonase telah ditentukan menggunakan rekabentuk pecahan pemfaktoran dua-peringkat. Proses pemilihan enam faktor; nilai pH, masa pengeraman, suhu, kepekatan pektin, saiz inokulum dan kelembapan medium, yang mempengaruhi penghasilan ekso-poligalakturonase oleh *A. niger* telah dilakukan. Keputusan analisis varians (ANOVA) mencadangkan empat faktor yang signifikan secara statistik dalam penghasilan ekso-poligalakturonase oleh *A. niger*. Faktor-faktor ini ialah masa pengeraman, suhu, kepekatan pektin dan kelembapan medium. Analisis statistik menunjukkan model matematik linear signifikan dengan nilai pekali penentuan (R^2) bersamaan dengan 0.9711. Penghasilan optimum ekso-poligalakturonase yang diperolehi berdasarkan model dalam kajian ini ialah sebanyak 40.00 U/g.

Kata kunci: *Aspergillus niger*, ekso-poligalakturonase, reka bentuk pemfaktoran dua-peringkat, pektinase

1.0 INTRODUCTION

Pectinase is one of the commercial enzymes on the market and serves to decompose pectin, a type of polysaccharide substrate contained within the cell wall of a plant. This enzyme breaks the polygalacturonic acid into monogalacturonic acid by opening the glycosidic linkages [1]. Pectinase generally hydrolyzes the pectic material depending on its capability for using the substrate (pectin and pectic acid) as well as its operating mechanism (hydrolysis or trans-elimination) [2]. Pectinase is produced by various types of microorganisms that use pectin as a carbon source [3]. Various agricultural and agro-industrial wastes have been utilized as a carbon source to stimulate the production of pectinases by microorganisms [4]. The remainder of the agriculture and agro-industry is a complex natural ingredient consisting of three main components namely hemicellulose, lignin and cellulose. Generally, the three major components of the cell wall are also named as lignocellulose.

Examples of lignocellulose sources used in the production of pectin are bagasse, citrus pulp, wheat bran, rice bran, corn straw, grains, fruit skin and vegetable skin [5]. Lignocellulose from agricultural and agro-industrial wastes is rich in carbohydrate that can support growth of microorganisms and can also stimulate production of valuable enzymes, such as the pectinase enzymes in solid state fermentation [6]. Researches on pectinase productions using pectin extract from agricultural wastes such as orange peel and pomace have been performed extensively [7, 8]. In addition, previous study showed that a tropical fern, *Nephrolepis biserrata* contains high level of pectin [9], which makes it potential to be used as a carbon source for production of pectinase from microorganism.

Solid state fermentation has advantages over other fermentation processes such as simple technique, low capital investment, cheaper production of enzyme with better physiochemical properties, lower level of catabolite repression and better product recovery [10, 11]. Generally, solid state fermentation is defined as a process involving growth of microorganisms in solid particles with solid material between particle spaces filled with continuous gas phase [12]. Among the key factors affecting solid state fermentation are types of single microorganisms, inoculum age, and medium used for the production of secondary metabolites in the correct physiology to produce the desired product [13].

Many useful enzymes are produced using industrial fermentation of *Aspergillus niger*. The processes provide extracellular fungal enzymes and have been the basis to initiate the microbial enzyme production by solid state fermentation in industrial environment [14]. *A. niger* fermentation is "generally regarded as safe" for use in the food processing industry, as well as non-toxicogenic and non-toxicigenic by the United States Food and Drug Administration [15].

In literature, studies on the optimization process to produce exo-polygalacturonase from lignocellulose waste can be found easily. However, reports that addressed the screening process for the affecting factors in the production of exo-polygalacturonase as the preliminary step are close to be none.

Previously, pectin from *N. biserrata* leaves was successfully extracted and optimized [9]. In this study, the *N. biserrata*'s pectin was used as a carbon source for production of exo-polygalacturonase in solid state fermentation of *A. niger*. Thus, the objective of this study is to screen the affecting factors in the production of exo-polygalacturonase using pectin extracted from *N. biserrata* leaves. There were total of six factors considered and the screening process were conducted using two-level fractional factorial design.

2.0 METHODOLOGY

2.1 Microorganisms and Inoculum Preparation

Aspergillus niger ATCC 120120 from glycerol culture stock was *revived* by plating on potato dextrose agar (PDA, Difco). The plate was casted at room temperature (27°C ± 1) for 7 days to allow mycelium growth. The spores were scrapped out and suspended in 0.1% w/v Tween-80. The mixture was centrifuged at 4,000 rpm at 4°C for 20 minutes to separate spores from lubricating solutions. The total number of spores from *Aspergillus niger* was calculated using a hemocytometer (Neubauer). 10% (v/v) of spore solution containing 1 x 10^7 spores/mL was used as inoculum.

2.2 Medium for Exo-polygalacturonase Production

Fermentation medium to produce exo-polygalacturonase by *Aspergillus niger* was prepared as described by Suresh *et al*. [17] using *Nephrolepis biserrata* pectin substrate which was extracted using method described previously [16]. The medium composition was 0.1 g/L $(NH_4)SO_4$, 0.5 g/L $MgSO_4.7H_2O$, 0.5 g/L KH_2PO_4, 0.005 g/L $FeSO_4.7H_2O$, 1.0 g/L yeast extract, 10.0 g/L glucose, and 10.0 g/L pectin of *Nephrolepis biserrata* leaves. The medium was sterilized in an autoclave for 1 hour at 121°C and cooled to room temperature (27°C ± 1) before inoculation with the fungus.

2.3 Exo-polygalacturonase Production in Solid-state Fermentation

The solid-state fermentation experiments were designed using two-level factorial design. Each experiment was carried out in 500 mL Erlenmeyer flasks, with 10 g of solid medium. 0.05 M citrate buffer, pH 5.0, was added to the medium until the moisture

content reaches 50% to 70%. The moisture content was measured using a moisture content analyzer (AND MX-50). The medium was sterilized and cooled before inoculation with 10% (v/v) *Aspergillus niger* inoculum containing 10^6 to 10^{10} spores/mL. After mixing uniformly, the flask was incubated in incubator at temperature and time according to the experimental design (Table 1). The experiments were performed in duplicate. The exo-polygalacturonase activity and biomass of *Aspergillus niger* were analyzed in each run. Sampling was performed every day.

2.4 Enzyme Analysis

One gram of fermented sample was collected every 24 hours. The sample was mixed with 10 mL citric-phosphate buffer (pH 5.0). The mixture was let suspended for 1 minute to ensure that all enzymes and sugars were extracted from the media to the buffer. Next, the suspended matter was centrifuged at 4,000 rpm, 4°C for 30 minutes to separate solid (pellet) from the liquid (supernatant or raw enzyme). The supernatant was subjected to analysis of exo-polygalacturonase enzyme activity. Determination of enzyme activity was carried out using dinitrosalicylic acid (DNS) with minimal modification. This method attempted to determine the carbonyl group in the reducing sugar [18]. The enzyme activity was identified by measuring the optical density after the reaction with DNS using spectrophotometer at wavelength of 540 nm [19]. One unit of exo-polygalacturonase activity is defined as the number of µM of reduced sugar measured in terms of glucose, produced as a result of the action of 1.0 ml of enzyme extract in 1 minute at 35°C ± 1°C [20].

Table 1 Factors in actual values for screening process by two-level factorial design

	Variables	Unit	Low level (-)	High level (+)
A	pH	-	3	7
B	Incubation time	Day	3	7
C	Temperature	℃	30	40
D	Pectin concentration	g/L	10	20
E	Inoculum size	*Log* spore	6	10
F	Moisture content	%	50	80

2.5 Determination of *Aspergillus niger Biomass*

Determination of biomass was conducted by 'Indirect Biomass Estimation' method described by Ramachandran *et al*. [21]. Estimation of fungal biomass was done by determining N-acetyl glucosamine released with acid hydrolysis of chitin found in the fungal wall cells. A total of 0.5 g of fermented sample was mixed with concentrated sulfuric acid (2 mL) in 500 mL conical flask and keep for 24 hours at 30°C. Then, the mixture was diluted with distilled water to make 1 N solution and was autoclaved (15 psi for 1 hour at 121°C) and then filtered with filter paper. The solution was then neutralized using 4 N NaOH solution and distilled water was added to 100 mL. A total of 1 mL of the solution was mixed with 1 mL of acetone acetylene and incubated in boiling water for 20 minutes. After cooling, 6 mL ethanol was added, followed by 1 mL Ehrlich reagent and incubated at 65°C for 10 minutes. After cooling, the optical density of the reaction mixture was read by spectrophotometer at 530 nm spanning distance to standard reagent (Glucosamine from Sigma). The results were expressed as mg glucosamine per gram of dry substrate fermentation (mg/gdsf).

2.6 Experimental Design

The factors that influenced the exopolymer production were screened using two-level factorial design by DESIGN EXPERT software (State-Ease Inc., Statistic made easy, Minneapolis, MN, USA, Version 6.0.4). Six variable factors were chosen, which were pH (A), incubation time (B), temperature (C), pectin concentration (D), inoculum size (E) and moisture content (F), to evaluate their effects on exo-polygalacturonase enzyme production. The response variable was exo-polygalacturonase enzyme activity that indicates enzyme production. The variables having most significant effects on the activity were then identified using two-level fractional factorial design, where the total runs were only half from the full runs. Fractional factorial design was chosen in order to ease the screening process for a process with large number of variables without having to deal with a large number of experiments. The design contained a total of 38 experimental trials involving six replicates at centre points. Each independent variable was investigated duplicate at superior a high (+1) and a low (-1) level (Table 1). Runs of center points were included in the matrix and statistical analysis was used to identify the effect of each variable on exo-polygalacturonase activity. The runs were randomized for statistical reasons. The variables having major effects on exo-polygalacturonase activity were identified on the basis of confidence levels above 95% ($P < 0.05$). The significant factors and interactions identified from the half normal plot analysis were chosen for generating the first-order model for the response of texture after the effects and interactions were evaluated. The significance of linear effects of the six variables was evaluated by variance analysis (ANOVA). The coefficient of determination of R^2 and adjusted R^2 coefficient were used for evaluation of the fit of the model.

3.0 RESULTS AND DISCUSSION

3.1 Significant Factors Affecting Production of Exo-polygalacturonase

Two-level fractional factorial design was used as a screening method to determine which of the six factors significantly affect exo-polygalacturonase production in the solid-state fermentation process using *Nephrolepis biserrta* leaves as the substrate. The six potential factors that might be affecting exo-polygalacturonase production were identified from previous research in the literature. The design consisted of 38 experiments with six center points is shown in Table 2. Screening design was used to detect the factors or independent factors that have a higher impact on the response variable of exo-polygalacturonase activity.

Table 2 Exo-polygalacturonase yields from two-level factorial design of the screening process

Run	(A) pH	(B) Incubation time, day	(C) Temp, °C	(D) Pectin concentration, g/L	(E) Inoculum size, log spore	(F) Moisture content, %	(Y) Exo-polygalacturonase activity, U/g	
							Experimental	Model
1	5.00	5.00	35.00	15.00	8.00	65.00	28.63	29.44
2	7.00	7.00	40.00	20.00	10.00	80.00	27.32	27.79
3	3.00	3.00	40.00	20.00	6.00	50.00	26.63	26.31
4	7.0.0	7.00	30.00	10.00	10.00	80.00	28.55	28.86
5	3.00	7.00	30.00	10.00	10.00	50.00	29.97	30.06
6	3.00	3.00	40.00	10.00	6.00	80.00	32.31	32.52
7	7.00	3.00	30.00	10.00	6.00	80.00	39.47	39.77
8	7.0.0	7.00	40.00	10.00	6.00	80.00	28.57	28.16
9	5.00	5.00	35.00	15.00	8.00	65.00	28.87	29.44
10	3.00	7.00	40.00	20.00	10.00	50.00	24.32	24.17
11	3.00	7.00	30.00	10.00	6.00	80.00	30.67	30.49
12	7.00	7.00	40.00	10.00	10.00	50.00	25.41	25,56
13	7.00	3.00	40.00	20.00	10.00	50.00	29.13	28.82
14	7.0.0	7.00	30.00	10.00	6.00	50.00	29.07	29.03
15	3.00	7.00	40.00	10.00	10.00	80.00	29.06	29.20
16	7.00	3.00	40.00	10.00	6.00	50.00	29.17	29.32
17	5.00	5.00	35.00	15.00	8.00	65.00	28.77	29.44
18	5.00	5.00	35.00	15.00	8.00	65.00	32.96	29.44
19	3.00	7.00	40.00	20.00	6.00	80.00	25.87	26.05
20	3.00	7.00	30.00	20.00	6.00	50.00	27.11	27.12
21	7.00	7.00	30.00	20.00	6.00	80.00	27.96	27.49
22	3.00	3.00	40.00	10.00	10.00	50.00	29.47	29.58
23	7.00	3.00	40.00	20.00	10.00	80.00	39.89	39.44
24	3.00	3.00	30.00	10.00	10.00	80.00	40.72	40.37
25	7.0.0	7.00	40.00	20.00	6.00	50.00	24.37	24.07
26	3.00	3.00	40.00	20.00	10.00	80.00	29.88	29.77
27	3.00	3.00	30.00	20.00	6.00	80.00	37.18	37.31
28	5.00	5.00	35.00	15.00	8.00	65.00	28.83	29.44
29	7.0.0	7.00	30.00	20.00	10.00	50.00	28.52	28.82
30	3.00	3.00	30.00	10.00	6.00	50.00	35.44	35.47
31	5.00	5.00	35.00	15.00	8.00	65.00	28.55	29.44
32	7.00	3.00	30.00	20.00	6.00	50.00	32.92	33.13
33	3.00	7.00	30.00	20.00	10.00	80.00	27.97	27.94
34	3.00	7.00	40.00	10.00	6.00	50.00	26.88	26.81
35	7.00	3.00	40.00	10.00	10.00	80.00	31.99	31.70
36	3.00	3.00	30.00	20.00	10.00	50.00	32.51	32.80
37	7.00	3.00	40.00	20.00	6.00	80.00	29.53	30.09
38	7.00	3.00	30.00	10.00	10.00	50.00	34.77	34.61

The interpretation of the study was analyzed statistically by using the variance analysis (ANOVA). P-value less than 0.05 indicates that the factor is significant to the responses studied. This is based on a confidence level that is set at 95%. The model is significant with the probability <0.0001. This means the regression model produced to explain the correlation of exo-polygalacturonase activity with the tested factors is exactly in statistics. ANOVA for exo-polygalacturonase activity using two-level factorial design is shown in Table 3. Based on the P values, it was identified that incubation time (B), temperature (C), pectin concentration (D) and moisture content (F) give significant effect on the enzyme production.

Table 3 Analysis of the variance (ANOVA) for exo-polygalacturonase activity using 2LFD

Source	Sum of squares	Degrees of freedom	Min square	Value > F	P>F
Model	580.22	19	30.56	30.08[a]	<0.0001[b]
A	0.013	1	0.013	0.014	0.9073
B	249.71	1	249.71	245.75	<0.0001
C	165.67	1	165.57	163.04	<0.0001
D	28.89	1	28.89	28.44	<0.0001
E	1.25	1	1.25	1.32	0.2663
F	53.17	1	53.17	56.04	<0.0001
AD	7.69	1	7.69	7.57	0.0136
BC	42.25	1	42.25	41.58	<0.0001
BF	13.27	1	13.27	13.06	0.0021
ADE	14.67	1	14.67	14.44	0.0014
Curvature	4.65	1	4.65	4.58	0.0471[c]
Residue	17.27	17	1.02	-	-
Inequality	2.29	12	0.19	0.064	0.9999[d]
Absolute error	14.98	5	3.00	-	-
Correlation error	602.58	37	-	-	-

[a] F-value is significant. [b] Model is significant, with P>F lower than 0.05.
[c] Significant curvature. [d] Model is fit due to insignificant F-value.
Standard deviation is 1.01

The relationship between all factors toward exo-polygalacturonase activity was shown by a regression equation generated from the statistical analysis (Equation 1). The fitness of the model was expressed by the R^2 value which was 0.9711, indicating that 97.11% of the variability in the response can be explained by the linear model.

Exo-polygalacturonase activity (U/g) = 30.39 + 0.020A – 2.79B – 2.28C – 0.95D + 0.20E + 1.29F – 0.15AB + 0.047AC + 0.49AD + 0.085AE – 0.044AF + 1.15BC + 0.029BD - 0.64BF – 0.038CD + 5.31CE + 0.30DE – 0.012ACE + 0.68ADE (1)

The half normal plot was used to determine the significant factors affecting the response. As shown in the half-normal plot (Figure 1), the factors that deviate far from the linear line are the significant factors that affect the production of exo-polygalacturonase. The effect of incubation time (B factor), temperature (C factor), pectin concentration (D factor) and substrate moisture content (F factor) are clearly deviated far from the linear line and thus indicate a strong signal. This result is concurrent with the P-values obtained in ANOVA. The next significant factors are the interaction factor between pH and pectin concentration (AD factor), interaction between incubation time and temperature (BC factor), interaction between incubation time and substrate moisture content (BF factor), and interaction between pH, pectin concentration and substrate moisture content (ADE factor).

Incubation time is one of the most important and highly significant variables obtained from the statistical analysis result of this study. It is apparent that the incubation period for this fermentation process affects the growth rate of the microorganisms and thus, the synthesis of enzymes. The incubation time allows the enzymes to react with the substrate at any given time where the reaction is more concentrated to produce the product effectively [23].

Temperature is also the significant factor identified in this statistical analysis. The importance of temperature in the development of biological processes is very evident as temperature effects protein folding, affects enzyme inhibition, promotes or inhibits certain metabolites, and it also determines the cellular properties and cell death [24]. Pectin concentration affects the enzyme production as it serves as the substrate to the enzyme. This is supported by Pandey [25], which stated that most exo-polygalacturonase is an indigenous enzyme which production is induced by the presence of pectin in the growth medium.

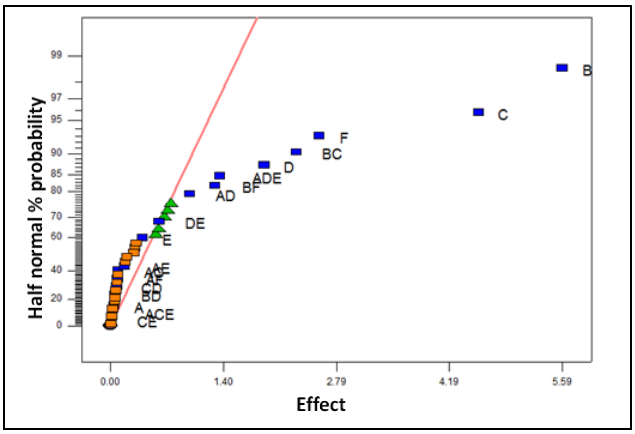


Figure 1 The half normal plot for the effect of pH (A), incubation time (B), temperature (C), pectin concentration (D), inoculum size (E) and moisture content (F)

Moisture content is one of the most important factors in solid state fermentation and it has also been identified to give significant effect in the production of pectinase [26]. In solid state fermentation process, high moisture content level will cause lower oxygen diffusion and lead to a reduction in gas exchange [27]. However, low level moisture content will also lead to a reduction in nutrient absorption, microbial growth, enzyme stability, and substrate development [13]. Therefore, moisture content is a significant factor where the optimum level required in the solid-state fermentation process needs to be determined.

3.2 Response Analysis of Exo-polygalacturonase Activity

The effect of each major factor as well as interaction among factors are statistically investigated as well as the effects of these variables toward response variable. As discussed in the previous section, four major variables (incubation time (B), temperature (C), pectin concentration (D) and moisture content (F)) have a very significant statistical effect on the production of exo-polygalacturonase. pH (factor A) and inoculum size (factor E) were found to be insignificant. These factors were statistically not affecting the solid-state fermentation process for the production of exo-polygalacturonase in this study. However, there is a significant interaction between pH and pectin concentration. Exo-polygalacturonase activity was found higher in pectin concentration of 10 g/L at pH 3 compared to pH 7. On the contrary, in pectin concentration of 20 g/L, there was an increase in exo-polygalacturonase activity at pH 3 to pH 7.

A significant interaction between temperature and incubation time was also identified. At temperatures ranging from 30℃ to 40℃, there was an increased activity of exo-polygalacturonase at incubation time of 3 days compared to incubation time of 7 days. The interaction between incubation time and moisture content in the range studied was also significant to the response of exo-polygalacturonase produced. In the medium with moisture content of 50% to 80%, the exo-polygalacturonase activity at the incubation time of 3 days was higher compared to incubation time at 7 days.

In addition to the impacts of major variables, interaction between these variables also show significant effects toward the response variable. Figure 2 shows the effects of interactions between two significant variables on the production of the exo-polygalacturonase. The interactions of AD (pH with pectin concentration) and BC (incubation time with temperature) have a significant effect on the response variable with P values less than 0.0001. The interactions of BF (incubation medium with medium humidity) as well as ADE (pH with pectin concentration, humidity and inoculum) also have a significant effect on the response variable with P values smaller than 0.05. Meanwhile, factors A (pH), E (inoculum size), and interactions of AB, AC, AE, AF, BD, CD, CE, DE and ACE are not significant to the response variable, with P values greater than 0.05. The effects of interaction between variables have also been reported by Seifollah *et al*. [28], whereby an increase in the production of exo-polygalacturonase was observed with increased concentration of hydrogen phosphate dihydrogen ranging from 0.4% to 0.8% with increment of incubation time ranging from 24 hours to 48 hours.

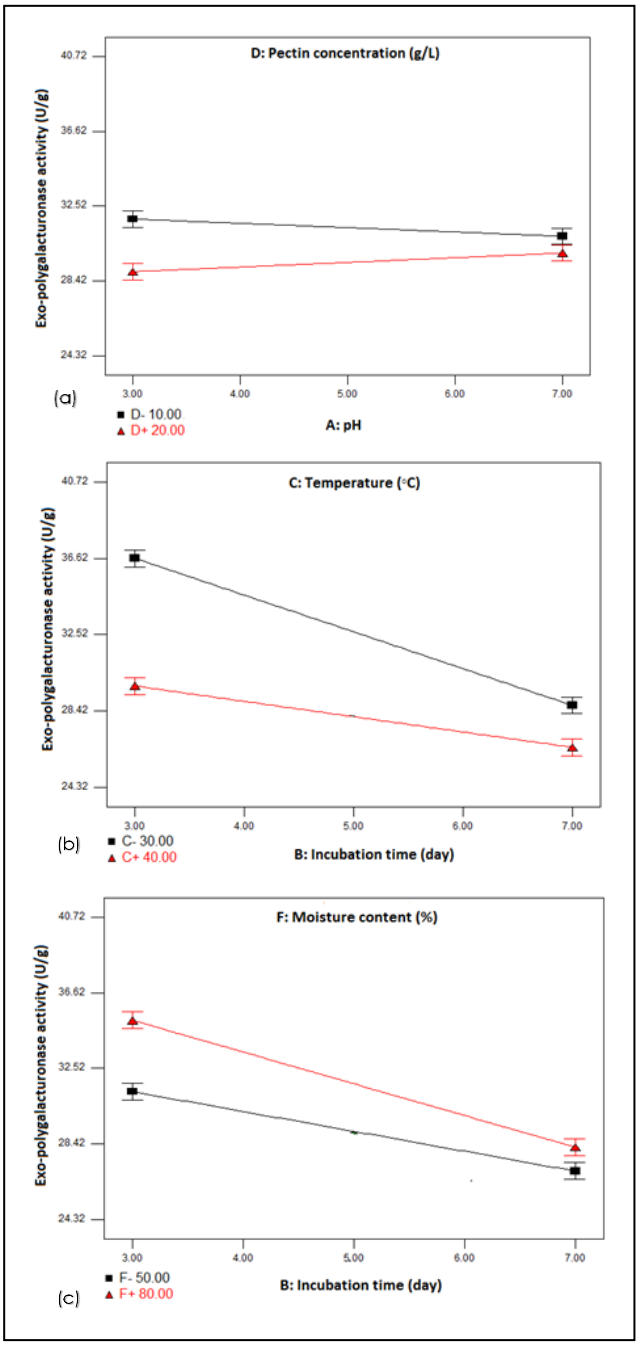


Figure 2 Plot of significant interaction factor between (a) incubation time - temperature, (b) pH - Pectin concentration, (c) with Incubation Time – Moisture Content to Exo-polygalacturonase activity

From the analysis, all the significant factors contributing to the production of exo-polygalacturonase by *Aspergillus niger* with activity of 40.00 U/g was obtained using the model. The results of this experiment suggest that the proposed mathematical model can explain the capabilities of the factors studied with the determination coefficient at 0.9711 (R^2 = 97.11%).

4.0 CONCLUSION

Two-level fractional factorial design has been used to screen for significant factors that affect exo-polygalacturonase production by *A. niger* in solid state fermentation using *N. biserrata* leaves as a substrate. Exo-polygalacturonase production was significantly affected by incubation time, temperature, pectin concentration and moisture content ($P < 0.0001$). The statistical analysis also shows that there are significant interactions between pH and pectin concentration, temperature and incubation time and pH, pectin concentration and inoculum size ($P < 0.05$) that affecting the production of exo-polygalacturonase. The proposed regression equation provides a fit mathematical model with R^2 at 0.9711 to evaluate the interactions between factors and further predict the production of exo-polygalacturonase.

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